more than one DNA sequence selected from the group consisting of a DNA sequence encoding a Dengue virus serotype 1 NS-1 antigen, a DNA sequence encoding a Dengue virus serotype 2 NS-1 antigen, a DNA sequence encoding a Dengue virus serotype 3 NS-1 antigen, and a DNA sequence encoding a Dengue virus serotype 4 NS-1 antigen.

OS Cond.

38. (Amended) The recombinant Modified Vaccinia Ankara (MVA) virus of Claim 37 comprising a DNA sequence encoding a Dengue virus serotype 1 NS-1 antigen, a DNA sequence encoding a Dengue virus serotype 2 NS-1 antigen, a DNA sequence encoding a Dengue virus serotype 3 NS-1 antigen, and a DNA sequence encoding a Dengue virus serotype 4 NS-1 antigen.

REMARKS

Claims 15-38 were pending in this application. Claims 29-31 have been cancelled without prejudice. A marked up version of the amended claims is provided in Appendix I. Following entry of the amendments claims 15-28 and 32-38 will be pending and at issue. A clean copy of all claims pending with this response is provided in Appendix II. Reconsideration of the application in view of the above amendments and following remarks is respectfully requested.

ALLOWABLE SUBJECT MATTER

Applicant acknowledges the Examiner's allowance of Claims 27, 28, and 32.

SUPPORT FOR AMENDMENTS TO THE CLAIMS

Claim 15 has been amended to include the term "homologous" to more clearly define Applicant's invention: a single recombinant MVA virus comprising more than one homologous DNA sequence from more than one Dengue virus serotype, and use of the said recombinant virus as a vaccine. Support for the term "homologous" can be found throughout the specification as filed, e.g., page 9, last two paragraphs.

Claim 18, 27, 33, 37, and 38 are amended to correct inadvertent and/or typographical

errors.

The amendments to the claims therefore add no new matter

REJECTIONS UNDER 35 U.S.C. § 112, FIRST PARAGRAPH

Claims 29-31 are rejected under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. Without agreeing with the Examiner's rejection but to expedite prosecution of this application, Applicant has cancelled Claims 29-31.

REJECTIONS UNDER 35 U.S.C. § 103 OF CLAIMS 15-17, 19-26, 35, 36

Claims 15-17, 19-26, 35 and 36 are rejected under 35 U.S.C. 103(a) as allegedly being unpatentable over either Sutter et al (C2) or Altenburger (US 5,185,146), in view of Lai et al (US 5,494,671), and further in view of either Monath et al (Fields Virology) or Kelly et al (US 6,074,865), and any of Moss (Seminars in Immunology 2:317-327, 1990) or Paoletti et al (WO 92/15672) or Nazerian et al (US 5,369,025). Applicant traverses this ground of rejection by amendment and argument.

Three requirements must be met for a prima facie case of obviousness. First, the prior art references must teach all the limitations of the claims. Second, there must be a motivation to modify the reference or combine the teachings to produce the claimed invention. Third, a reasonable expectation of success is required. Applicant argues that the art cited by the Examiner does not teach each and every element of Applicant's invention, specifically a <u>single</u> MVA vector comprising two or more <u>homologous</u> DNA sequences coding for antigens from two or more Dengue virus serotypes. Applicant also argues that the prior art teaches that there was no reasonable expectation of success of combining a <u>single</u> MVA vector with two or more <u>homologous</u> sequences coding for antigens from two or more Dengue virus serotypes. Applicant notes that the Examiner stated that, in view of the combined teachings of the references, the invention as a whole is allegedly obvious <u>absent unexpected results</u>.

The instantly claimed invention is based on a discovery by the Applicant that vaccination with a recombinant MVA virus expressing the same antigen from more than one Dengue virus serotype (e.g., at least two homologous antigens, each from a different serotype) results in immunity against all four serotypes of Dengue virus. Claim 15 has been amended to more clearly recite the invention by addition of the term "homologous." Claim 15 is directed to a single recombinant MVA virus comprising more than one homologous DNA sequence selected from a group consisting of DNA sequences coding for the same antigens from all four Dengue virus serotypes. Claims 16, 17, and 19-26 are dependent on claim 15 and therefore also include this limitation. Claims 35 and 36 are directed to a recombinant MVA virus comprising DNA sequence coding for E antigens from at least two (Claim 35) and all four (Claim 36) Dengue virus serotype. Therefore, Claims 35 and 36 are also directed to MVA virus comprising more that one homologous DNA sequence (i.e., E antigen) from different Dengue virus serotypes.

The cited prior art references do not teach all of the elements of the claims. Sutter et al relates to the use of a recombinant vaccinia vector system as an expression vector. Likewise, Altenburger et al relates to the use of MVA as an expression system. Sutter et al and Altenburger et al generally relate to the use of expression vectors based on vaccinia virus. Moss is a general reference relating to the use of recombinant DNA virus vectors for vaccination. Although these references teach using a MVA vector, and using an MVA vector for vaccination, none teaches a single MVA vector comprising more than one homologous DNA sequences from different Dengue serotypes

Neither Lai et al nor Kelly et al teaches a viral based vector comprising more than one homologous Dengue virus sequence. Lai et al describes a vaccinia virus expressing only a single serotype 4 dengue antigen. This virus can be used as vaccine, which is suitable to induce an immune response only against dengue virus serotype 4. Similarly, Kelly et al relates to a recombinant fragment of a single dengue virus serotype (serotype 2) expressed in a baculovirus; the purified protein expressed in baculovirus is used as antigen for a vaccine. These references each teach a viral based vector comprising a single DNA sequence from one Dengue virus serotype.

The Examiner notes that Kelly et al suggests the use of a tetravalent vaccine containing antigens from all four Dengue virus serotypes. However, Kelly teaches using purified protein expressed in baculovirus as antigen; one of skill in the art would assume that Kelly's suggestion regarding a tetravalent vaccine is directed to a vaccine of four purified proteins and not a vaccine of four DNA sequences. Therefore Kelly et al does not teach or suggest a single viral vector comprising more than one homologous Dengue virus DNA sequence.

Accordingly, the combination of Sutter et al and/or Altenburger et al and/or Moss and/or Lai et al and/or Kelly et al does not include the element of more than one homologous Dengue virus DNA sequence. The combination cannot render the claims obvious.

Monath et al does not remedy this deficiency. Monath et al relates to vaccination with live, attenuated dengue viruses of all four serotypes in order to avoid immune enhancement or antibody dependent enhancement. The Examiner states that Monath provides motivation for including multiple dengue serotypes simultaneously. However, Monath does not teach (or provide motivation for) combining more than one isolated, homologous DNA sequence from different Dengue serotypes in a single viral vector. Instead Monath teaches using complete dengue viruses; in fact, Monath et al notes that induction of immune enhancement or antibody dependent enhancement is a serious concern for the use of subunit vaccines (page 1003, column 1, last paragraph) and arguably teaches away from using isolated, homologous DNA sequences.

Both Paoletti et al and Nazarian et al relate to viral vectors comprising more than one DNA sequence from more than one viral serotype. However, neither reference teaches more than one homologous DNA sequence from more than one viral serotype. Paoletti et al relates to the expression of hemagglutinin genes of three serotypes of *avian influenza virus* in a fowlpox virus. Hemagglutinin encoding sequences of influenza viruses are extremely variable encoding 14 different serotypes, wherein the nucleotide sequences of the serotypes vary not only in base composition but also in length. Applicant respectfully directs Examiner's attention to page 1359 from the textbook Fields et al, Virology (1996) 3rd edition, Lippincott-Raven Publishers, Philadelphia attached to this response as Appendix V ("Fields"). Turning the right column the

authors note:

> . . .Since then the nucleotide sequences of RNA segment 4 of all known 14 known HA antigenic subtypes and many variants with a subtype have been determined. RNA segment 4 ranges from 1742 to 1778 nucleotides and encodes a polypeptide of 562 to 566 residues.

Therefore, the hemagglutinin genes are not homologous.

Nazarian et al relates to a fowlpox virus recombinant for a sequence encoding the glycoprotein B homologue (gBh) of Marek's disease virus (MDV); Nazarian proposes insertion of several different immunogenic genes of MDV into the viral vector. The different MDV genes are not homologous with each other, but rather are named according to their homology to HSV genes. Therefore, neither Paoletti et al nor Nazarian et al teach more than one homologous DNA sequence from more than one viral serotype, and neither remedies the deficiencies of the combination of prior art described above.

One of skill in the art would have had no expectation of success. Assuming that the combination of art cited against Claims 15-17, 19-26, 35 and 36 does contain all the claim elements (and Applicant does not concede that it does), one of skill in the art would have had no expectation of success when combining the elements. First, homologous sequences in a single MVA vector were thought to be unstable. Second, immunization against more than one Dengue virus serotype using a single antigen was thought to be dangerous due to immune and/or antibody enhancement.

Homologous sequences in a single poxviral vector were thought to be unstable. In the present invention, more than one homologous DNA sequences (e.g., a PreM Dengue virus sequence that is 71-77% homologous between the 4 Dengue virus serotypes) are inserted into a single poxviral vector (e.g., MVA). Prior to Applicant's invention, one of skill in the art would have expected that a poxviral vector comprising several homologous sequences would be unstable. Applicant respectfully directs the Examiner's attention to the reference Howley et al (1996) Gene 172: 233-237, attached to this Response as Appendix III ("Howley"). According to

Howley, the insertion of two or three identical sequences into a vaccinia into a vaccinia based vector results in recombination between the homologous sequences. See Howley, Abstract and page 237, left column.

> Insertion of genes with VV (vaccinia virus) p7.5 promoters into the I4L, J2R, and K1L loci of the same virus produced viable virus recombinants <u>even though recombination between these loci could be demonstrated</u>.

Thus, one of skill would have expected that homologous recombination would occur in a single poxviral vector comprising more than one homologous sequence.

The MVA vector used in the instant application is unstable even without inserted homologous sequences. Applicant respectfully directs the Examiner's attention to the reference Meyer et al (1991) Journal of General Virology 72: 1031-1038 attached to this Response as Appendix IV ("Meyer"). According to Meyer, MVA is a highly attenuated virus derived from the vaccinia virus strain Ankara (CVA) by continuous passaging in chicken embryo fibroblasts. During this continuous passaging the pox virus becomes unstable and several major deletions in the genome were accumulated. See Meyer, page 1036, right column, lines 2-4, noting "complex sequence rearrangements or transpositions" and various deletions.

One of skill in the art would not expect success when combing the unstable MVA vector with the destabilizing effect of multiple homologous sequences in a poxviral vector. One of skill would expect unpredictable and unstable results. The possible results include loss of one of the homologous sequences and a vector comprising only one antigen sequence. Due to the possibility of immune and/or antibody enhancement when using a single Dengue virus serotype (see below) the use a such a vector for a vaccine could be dangerous.

Immunization against more than one Dengue virus serotype was thought to result in serious complications. As discussed in Applicant's earlier response, immunization of a subject against one Dengue virus subtype may result in antibody-dependent enhancement and/ or immune enhancement, when the subject is later infected with a different Dengue virus serotype.

Based on this information, prior to Applicant's invention, the skilled artisan would have believed that vaccination with the same antigen from tow or more Dengue virus serotypes, e.g., homologous DNA sequences from two or more Dengue virus serotypes, would result in severe antibody-dependent enhancement and/ or immune enhancement. Indeed, prior art cited by the Examiner, Monath et al, teaches that it is essential to use the complete Dengue viruses for vaccination to provide a sufficient diversity of antibodies, in order to avoid antibody-dependent enhancement and/ or immune enhancement complications that arise from the use of subunit vaccines. For example, see Monath at page 1003, column 1, last paragraph.

Thus, Applicant's discovery that vaccination with a recombinant MVA virus expressing more than one homologous Dengue virus serotype antigen (e.g., the same antigen from two or more Dengue virus serotypes) results in immunity against all four serotypes of Dengue virus without complications was an unexpected success.

In conclusion, the combination of Sutter et al and/or Altenburger et al and/or Moss and/or Lai et al and/or Kelly et al and/or Monath et al and/or Paolotti et al and/or Nazerian et al does not include the all the elements of Claim 15, e.g., a single MVA virus comprising more than one Dengue virus DNA sequence wherein the sequences are homologous. Further, the combination of elements in Applicant's invention is an unexpected success in view of the prior art teaching that MVA is an unstable vector made more unstable by the addition of homologous sequences. Therefore, a prima facie case of obviousness is not made. Withdrawal of this ground of rejection of **Claims 15-17, 19-26, 35 and 36** is respectfully requested.

REJECTIONS UNDER 35 U.S.C. § 103 OF CLAIMS 18 AND 33-38

Claim 18 was rejected under 35 U.S.C. 103(a) as being allegedly unpatentable over either Sutter et al (C2) or Altenburger (US 5,185,146), in view of Lai et al (US 5,494,671), and either Monath et al (Fields Virology) or Kelly et al (US 6,074,865), and any of Moss or Paoletti et al (WO 92/15672) or Nazerian et al (US 5,369,025), as applied to claims 15-17 and 19-26 above, and further in view of further in view of Sutter et al (PNAS 89:10847-1085 1, 1992).

Claims 33-38 were rejected under 35 U.S.C. 103(a) as being allegedly unpatentable over either Sutter et al (C2) or Altenburger (US 5,185,146), in view of Paoletti et al (5,744,141 and 5,514,375) and either Monath et al (Fields Virology) or Kelly et al (US 6,074,865), and any of Moss or Paoletti et al (WO 92/15672) or Nazerian et al (US 5,369,025).

Applicant traverses these rejections for the reasons stated above in response to the rejection of Claims 15-17, 19-26, 35 and 36. Claim 18 is dependent on Claim 15 and includes the same limitations: a single MVA virus comprising more than one homologous DNA sequence from more than one Dengue virus serotype. Claims 33-38 also include these limitations. The combination of art cited against claim 18 and claims 33-38 includes the art cited against Claims 15-17, 19-26, 35 and 36. As discussed below, the additional art cited does not remedy the deficiencies regarding the missing claims elements, and does not provide support to rebut Applicant's argument of unexpected success.

Sutter et al (additional art cited for Claim 18) relates to the use of recombinant MVA virus as a vector for heterologous gene expression. Sutter et al does not teach or suggest a recombinant MVA virus comprising more than one homologous Dengue virus serotype antigen DNA sequence. Accordingly, Sutter et al does not remedy the deficiency of the combination of Sutter et al and/or Altenburger et al and/or Moss and/or Lai et al and/or Kelly et al and/or Monath et al and/or Paolotti et al and/or Nazerian et al. Accordingly, the combination cannot render the claimed invention obvious. Withdrawal of this ground of rejection is respectfully requested.

Paoletti et al (5,744,141 and 5,514,375, additional art cited against Claims 33-38) relates to a recombinant vaccinia comprising the entire preM, E, and NS-1 dengue virus coding sequences. Paoletti et al does not teach or suggest a recombinant MVA virus comprising more than one homologous Dengue virus serotype antigen DNA sequence. Accordingly, Paoletti et al does not remedy the deficiency of the combination of Sutter et al and/or Altenburger et al and/or Moss and/or Kelly et al and/or Monath et al and/or Paolotti et al and/or Nazerian et al. Accordingly, the combination cannot render the claimed invention obvious. Withdrawal of this

ground of rejection is respectfully requested.

CONCLUSION

Withdrawal of the pending rejections and reconsideration of the claims are respectfully requested, and a notice of allowance is earnestly solicited. If the Examiner has any questions concerning this Response, the Examiner is respectfully requested to telephone Applicant's representative at (415) 393-2654.

NOTICE OF FIRM NAME CHANGE

Agent for Applicant wishes to inform the Office that the name of its firm has been changed to Bingham McCutchen LLP.

In addition, this response is accompanied by an Associate Power of Agent/Attorney for Susan Hubl, Ph.D., Patent Agent, to whom all correspondence should be directed.

Respectfully submitted,

Dated: November 1, 2002

By: [signature]

Susan Hubl, Ph.D., Patent Agent
Registration No.: 47,668

Mailing Address:

Bingham McCutchen, LLP
Three Embarcadero Center
San Francisco, California 94111
Telephone: 415.393.2000
Telefax: 415.393.3386

Appendix I

Version of the Amendments to the Claims with Markings to Show Changes Made

15. (Amended) A recombinant Modified Vaccinia Ankara (MVA) virus comprising more than one homologous DNA sequence selected from the group consisting of a DNA sequence encoding a Dengue virus serotype 1 antigen, a DNA sequence encoding a Dengue virus serotype 2 antigen, a DNA sequence encoding a Dengue virus serotype 3 antigen, and a DNA sequence encoding a Dengue virus serotype 4 antigen.

18. (Amended) The recombinant MVA virus according to Claim 15, wherein the DNA sequences are~~-is~~-inserted at the site of one or more naturally occurring deletions within the MVA virus genome.

27. (Amended) (Allowed) A composition comprising a first and second component, wherein the first component is a vector comprising more than one DNA sequence selected from the group consisting of a DNA sequence encoding a Dengue virus serotype 1 antigen, a DNA sequence encoding a Dengue virus serotype 2 antigen, a DNA sequence encoding a Dengue virus serotype 3 antigen, or a DNA sequence encoding a Dengue virus serotype 4 antigen and wherein the more than one DNA sequences are under the transcriptional control of a T7 RNA polymerase promoter and the second component is a recombinant Modified Vaccinia Ankara (MVA) virus comprising a DNA sequence encoding T7 RNA polymerase~~-and~~.

29. (Cancelled) ~~A method for mounting an immune response in an animal to Dengue virus infection, the method comprising administering to the animal the composition of Claim 27.~~

30. (Cancelled) ~~The method according to Claim 29, wherein the animal is a human.~~

31. (Cancelled) ~~The method of Claim 29 wherein the first component is administered prior to the second component. comprising more than one preDNA sequence selected from the group~~

33. (Amended) A recombinant Modified Vaccinia Ankara (MVA) virus comprising more than one DNA sequence selected from the group consisting of a DNA sequence encoding a Dengue virus serotype 1 preM antigen, a DNA sequence encoding a Dengue virus serotype 2 preM antigen, a DNA sequence encoding a Dengue virus serotype 3 preM antigen, and a DNA sequence encoding a Dengue virus serotype 4 preM antigen.

37. (Amended) A recombinant Modified Vaccinia Ankara (MVA) virus comprising more than one DNA sequence selected from the group consisting of a DNA sequence encoding a Dengue virus serotype 1 NS-1 antigen, a DNA sequence encoding a Dengue virus serotype 2 NS-1 antigen, a DNA sequence encoding a Dengue virus serotype 3 NS-1 antigen, and a DNA sequence encoding a Dengue virus serotype 4 NS-1 antigen.

38. (Amended) The recombinant Modified Vaccinia Ankara (MVA) virus of Claim ~~35~~ 37 comprising a DNA sequence encoding a Dengue virus serotype 1 NS-1 antigen, a DNA sequence encoding a Dengue virus serotype 2 NS-1 antigen, a DNA sequence encoding a Dengue virus serotype 3 NS-1 antigen, and a DNA sequence encoding a Dengue virus serotype 4 NS-1 antigen.

Appendix III

Howley et al (1996) Gene 172: 233-237

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Gene, 172 (1996) 233–237

0378-1119/96/$15.00

GENE 09789

A vaccinia virus transfer vector using a *GUS* reporter gene inserted into the *I4L* locus

(Poxvirus; viral vector; β-glucuronidase; ribonucleotide reductase)

Paul M. Howley, Danièle Spehner and Robert Drillien

INSERM U74, and Institut de Virologie de la Faculté de Médecine, Université Louis Pasteur, 67000 Strasbourg, France

USSN 09/147,919

ELSEVIER

Gene. 172 (1996) 233–237

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SUMMARY

A vaccinia virus (VV) transfer vector is described which enables integration of heterologous sequences into the *I4L* locus (ribonucleotide reductase—encoding gene) through co-insertion of a *GUS* selection marker. *I4L*⁻ VV recombinants formed blue plaques when an agarose overlay containing XGluc (5-bromo-4-chloro-3-indolyl-β-glucuronide) was added to the infected cell monolayer. Viruses already containing a *lacZ* reporter gene were also suitable recipients for the selection procedure since infection with a VV *lacZ* recombinant did not produce any blue plaques with XGluc. The addition of a synthetic early promoter downstream from the *GUS* cassette initiated the predicted-size transcript during an infection. Insertion of genes with VV *p7.5*-promoters into the *I4L*, *J2R* and *K1L* loci of the same virus produced viable virus recombinants even though recombination between these loci could be demonstrated. These techniques should be valuable for the further development of VV as a polyvalent vector.

INTRODUCTION

Vaccinia virus (VV), the prototype of the poxvirus family, has become a very useful and versatile mammalian expression vector which has been applied to solve fundamental problems in biology and in the development of recombinant (re-)DNA vaccines (Moss, 1990; 1992; Mackett, 1990). VV has a large (approx. 190 kb) double-stranded DNA genome containing a number of non-essential genes (Goebel et al., 1990; Johnson et al., 1993) into which foreign sequences fused to a VV promoter can be inserted by homologous recombination. The most popular site for insertion has been the thymidine kinase-encoding gene (*tk*, the *J2R* locus), for which the re-virus can be selected on tk^- cells in the presence of 5′-bromodeoxyuridine (Mackett et al., 1984)

Insertion into other non-essential VV genes is not associated with a convenient selection procedure. This obstacle can be overcome by inserting a selection marker along with the foreign gene; examples of such markers are the neomycin resistance (Franke et al., 1985) and the *Escherichia coli* (*Ec*) gene for guanine phosphoribosyl transferase (*gpt*) (Falkner and Moss, 1988; Boyle and Coupar, 1988). A very practical reporter gene has been the *Ec lacZ* gene, which enables blue plaque screening after the addition of XGal to the medium (Chakrabarti, 1985; Panicali et al., 1986).

Our interest has been the insertion of a foreign gene into a locus other than *tk* and the use of a selection

Correspondence to: Dr. R. Drillien, Laboratoire de Virologie U74 INSERM, 3 rue Koeberlé, 67000 Strasbourg, France. Tel. (33-88) 56 63 00; Fax (33-88) 56 63 03.

Abbreviations: bp, base pair(s); DIG, digoxigenin; *Ec*, *Escherichia coli*; GUS, β-glucuronidase, *GUS*, GUS-encoding gene; kb, kilobase(s) or 1000 bp; *I4L*, gene encoding large subunit of ribonucleotide reductase; m.o.i., multiplicity of infection; nt, nucleotide(s); *p*, promoter; pfu, plaque-forming unit(s); re-, recombinant; *tk*, VV gene encoding thymidine kinase; VV, vaccinia virus; wt, wild type; XGal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; XGluc, 5-bromo-4-chloro-3-indolyl-

precludes its use for the addition of other foreign genes. We have chosen to use the *I4L* locus (the gene encoding for the large subunit of ribonucleotide reductase) as a site of integration since it has been demonstrated that this gene is non-essential for viral replication in tissue culture (Child et al., 1990). These authors found that *I4L*⁻ recombinants produce plaques with the same morphology as the wild-type (wt) virus and that they do not require rapidly dividing cells to multiply, i.e., infection and multiplication was as efficient on confluent cell monolayers as on subconfluent cells. For the selection of an *I4L*⁻ re-virus, the *GUS* reporter gene encoding β-glucuronidase (GUS) was chosen (the GUS protein can breakdown the chromogenic substrate XGluc to produce a blue colour enabling easy detection of the presence of the gene). This gene reporter system has been widely used in transgenic plants (Jefferson, 1989) and it has also been successfully used in mammalian cells for the selection of human cytomegalovirus recombinants (Jones et al., 1991). Very recently the isolation of re-VV on the basis of selection for expression of *GUS* has also been reported (Carroll and Moss, 1995).

EXPERIMENTAL AND DISCUSSION

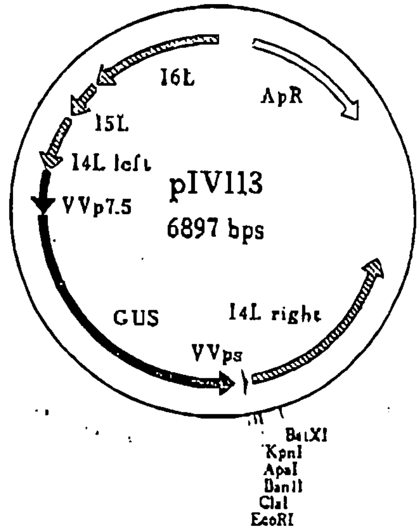
(a) Construction of VV transfer vector

The construction of our transfer vector containing *GUS* driven by a VV *p7.5*-promoter and flanked by VV *I4L* sequences is described in the legend of Fig. 1. This vector was named pIV110.

(b) Integration of the *GUS* cassette into the *I4L* locus of VV

The *GUS* gene was integrated into the *I4L* locus of wt VV by infecting 1×10^6 BHK21 cells with the Copenhagen strain at a m.o.i. of about 0.1 pfu/cell and transfecting with approximately 1 μg DNA from a mini-prep of pIV110 with LipofectAMINE (Gibco-BRL, Gaithersburg, MD, USA) as described by the manufacturer's instructions. Two days later the cells were recovered and the VV produced was plated after serial dilutions on fresh BHK21 cells. To detect viral recombinants producing GUS, infected cells were overlaid one day later with 1% low melting point agarose containing XGluc at a final concentration of 0.2 mg/ml. A number of blue stained plaques were picked and used to go through four cycles of plaque purification until 100% of the plaques displayed a blue colour. The new re-virus was designated VVIV110. An XGluc overlay added to BHK21 ... VVIV76 (a re-virus expressing

A



B

*Eco*RI AAAAATTGAAAAACTATTCTAATTTATTGCAC *Cla*I

Fig. 1. Plasmid map of pIV113 (A) and sequence of the synthetic promoter (B). A: VV*p7.5* is the VV *p7.5*-promoter; VV*ps* is the VV early synthetic promoter. VV ORFs (I4L, I5L and I6L) are depicted as hatched arrows. B: Synthetic promoter has *Eco*RI and *Cla*I sites at ends which were ligated into pBluescribe before cloning between the *Eco*RI and *Kpn*I sites of pIV110 to produce pIV113 (*Cla*I site of pIV110 can not be cut). The arrows indicate the major transcription start points determined by Davison and Moss (1989). Methods: To construct our transfer vectors, a 3.1-kb *Sal*I-*Bam*HI fragment overlapping a region of the VV *Hin*dIII-I fragment from the 3' end of *I4L* to the 5' end of *I6L* was subcloned between the *Xho*I and *Bam*HI sites of pPolyIII (Lathe et al., 1987) to produce clone pIV102. The *GUS* sequence from pGUSN358→S (Clontech, Palo Alto, CA, USA) was cut out with *Nco*I + *Eco*RI and cloned into the I4L segment of pIV102 using the same sites to produce pIV109. To express the *GUS* gene, the VV *p7.5*-promoter was joined to the 5' *GUS* sequence. First, pIV109 had to be modified by creating an *Nsi*I site by cutting the vector with *Nco*I, blunt ending with the Klenow fragment of *Ec* DNA polymerase and then self ligating to produce a unique *Nsi*I site. The VV *p7.5*-promoter from pTG2131 (Wild et al., 1992) was cloned into pIV109 by ligating the *Pst*I fragment containing the promoter into the *Nsi*I site thus producing pIV110. pIV113 was created by excising with *Kpn*I + *Eco*RI a double-stranded oligodeoxyribonucleotide sequence (B) that had been synthesized, annealed and ligated between the *Eco*RI and *Cla*I sites of Bluescript KS⁻ (Stratagene, La Jolla, CA, USA) and then cloned between the *Eco*RI and *Kpn*I sites of pIV110 thereby generating pIV113 (A). The Bluescript intermediate was necessary as the *Cla*I site of pIV110 would not cut even after transforming the vector into a *dam*⁻ strain of *Ec* (GM83). ApR, ampicillin-resistance marker.

To determine if the *GUS* reporter gene had successfully integrated into the *I4L* locus of the re-VVIV110 virus Southern blot hybridisation experiments were carried out on *Hin*dIII-digested DNA isolated from BHK21 infected

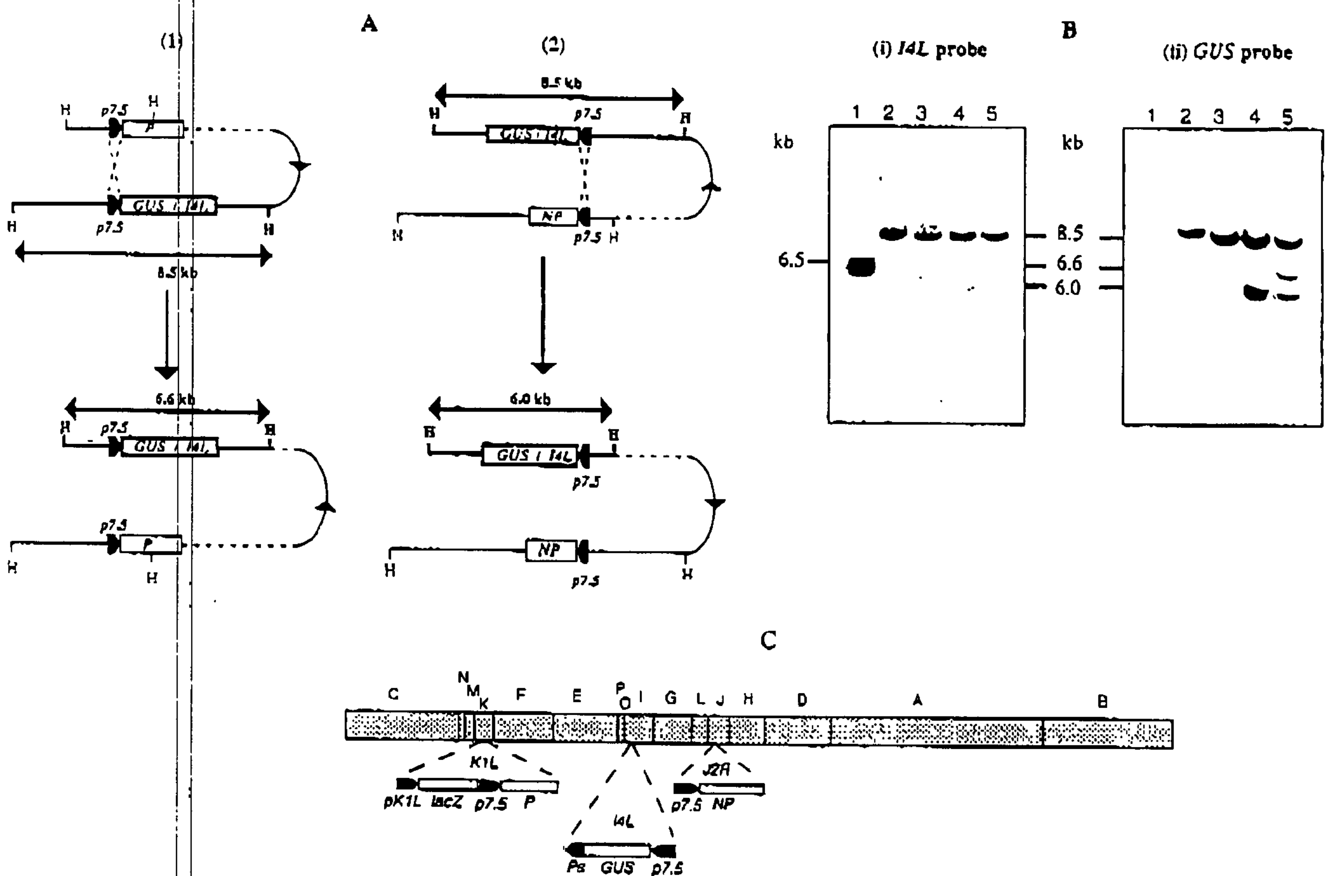


Fig. 2. Schematic map of the re-VV (C), the mechanism of the recombination events (A) and Southern analysis of the re-*Hin*dIII-I fragments (B). A: A schematic drawing of intrachromosomal recombination events between inversely oriented *p7.5*-promoters. Only the relevant portions of the VV genome with their characteristic *Hin*dIII (H) sites have been represented. The viral genomes have been folded so as to align the *p7.5*-promoters involved in the recombination. The diagrams showing the crossingover events give rise to the products which are shown below them. Note that after recombination the orientation of the intervening viral sequences (dashed line followed by curved line containing an arrowhead indicating an arbitrary orientation) is inverted and the 8.5-kb *Hin*dIII fragment containing *GUS* and part of the *I4L* gene becomes either a 6.6 or a 6.0-kb fragment (shown in bold in the diagrams). B: Southern blot of *Hin*dIII digested DNA extracted from BHK21 cells infected with different VV. Lanes: 1, VV Copenhagen strain; 2, VVIV110; 3, VVIV113; 4, VVIV113NP; 5, VVIV113NP/P. (i) was hybridised with DIG-labelled *I4L* DNA probe and (ii) was hybridised with DIG-labelled *GUS* DNA probe. **Methods:** Total DNA was isolated from 3×10^6 BHK21 infected cells. One tenth of the DNA samples was digested with 20 units of *Hin*dIII, then electrophoresed on an 0.8% agarose gel followed by Southern blotting onto a positively charged nylon membrane (Boehringer Mannheim, Mannhein, Germany). Hybridisation was carried out at 68°C with digoxigenin (DIG)-labelled *GUS* (*Nco*I-*Eco*RI fragment from pGUSN358→S) or DIG-labelled I4L (*Kpn*I-*Xho*I fragment from pIV110) DNA probes (Boehringer DNA labelling kit). Post hybridisation washes and the detection procedure were carried out as described by the manufacturer's instructions (Boehringer Luminescent detection kit). C: A schematic drawing of the VV genome showing the characteristic *Hin*dIII fragments and the positions and directions of *lacZ*, *GUS*, the measles virus *NP* and *P* genes.

*Hin*dIII-I fragment of VVIV110. The latter fragment (about 8.5 kb) can be accounted for by insertion of the 2.08-kb *GUS* sequence into the 6.5-kb *Hin*dIII-I fragment of the genome at the *I4L* locus. Fig. 2B(ii), lane 2 confirms the presence of the *GUS* gene within the enlarged *Hin*dIII-I fragment of the re-virus (note, no hybridisation to wt *Hin*dIII-I fragment; see lane 1 of Fig. 2B(ii)).

(c) Analysis of RNA transcripts initiated from a VV early synthetic promoter inserted down-stream of *GUS*

To see if it would be possible to obtain expression of *GUS* by linking its cDNA sequence to *GUS* we inserted downstream from *GUS* in pIV110 a strong early synthetic promoter (Davison and Moss, 1989). This created pIV113 (Fig. 1A) which was used to integrate *GUS* and the synthetic promoter into the *I4L* locus of the following VV by homologous recombination: VV wt strain, a re-VV encoding the measles virus nucleoprotein, VVNP (*NP* inserted into the *tk* gene), and a re-VV encoding both the measles virus nucleoprotein and phosphoprotein, VVNP/P (*P* gene inserted into the *K1L* locus with a *lacZ* reporter gene). Blue plaques were selected using XGluc, and the new recombinants were designated VVIV113, VVIV113NP and VVIV113NP/P.

To assay for early transcription from the synthetic promoter total RNA was isolated from BHK21 cells infected for 6 h with VVwt, VVIV110, VVIV113, VVIV113NP and VVIV113NP/P in the presence of cycloheximide (to block late transcription). Fig. 3 shows the results of the Northern hybridisation with an *I4L* probe. A major RNA transcript (2.7 kb) detected in the sample from the VVwt infection (lane 1) corresponds in size to the product of transcription from the natural *I4L* promoter (Tengelsen et al., 1988). A minor transcript found in samples marked with an arrowhead was derived from the readthrough of the *I4L* transcription stop signal and termination at the *I3L* transcriptional stop sequence. This readthrough was also observed by Tengelsen et al. (1988). Infection with VVIV110 gave rise to a major 4.7-kb transcript derived from the *I4L* promoter whose size was enlarged compared to the natural *I4L* transcript due to insertion of *GUS* and a 4.0-kb RNA species derived from transcription start at the *p7.5*-promoter (lane 2). Transcription from the latter promoter was generally weaker than expected and could be due to interference by the closely positioned *I4L* promoter. Infection with VVIV113, VVIV113NP and VVIV113NP/P (Fig. 3, lanes 3, 4 and 5) led to the synthesis of a 4.5-kb transcript from the *I4L* promoter and a 3.8-kb transcript from the *p7.5*-promoter; the size of each of these RNAs correlated well with the insertions that had been engineered into the *I4L* gene (Fig. 3B). In addition to these two transcripts the last three viruses induced the synthesis of a major RNA species (1.9 kb) originating through transcription from the synthetic promoter inserted downstream from *GUS*.

(d) Analysis of the recombination events involving multiple *p7.5*-promoters present in the re-VV

Viruses VVIV113, VVIV113NP, and VVIV113NP/P contained, respectively, one, two and three *p7.5*-promoters used to transcribe the *GUS*, *NP* and *P* genes. It should be noted that the VV Copenhagen strain used in this work does not contain a natural *p7.5 kDa* gene found in the WR strain (Goebel et al., 1990). The *p7.5*-promoter in front of *GUS* is located in the opposite orientation on the VV genome to the two *p7.5*-promoters

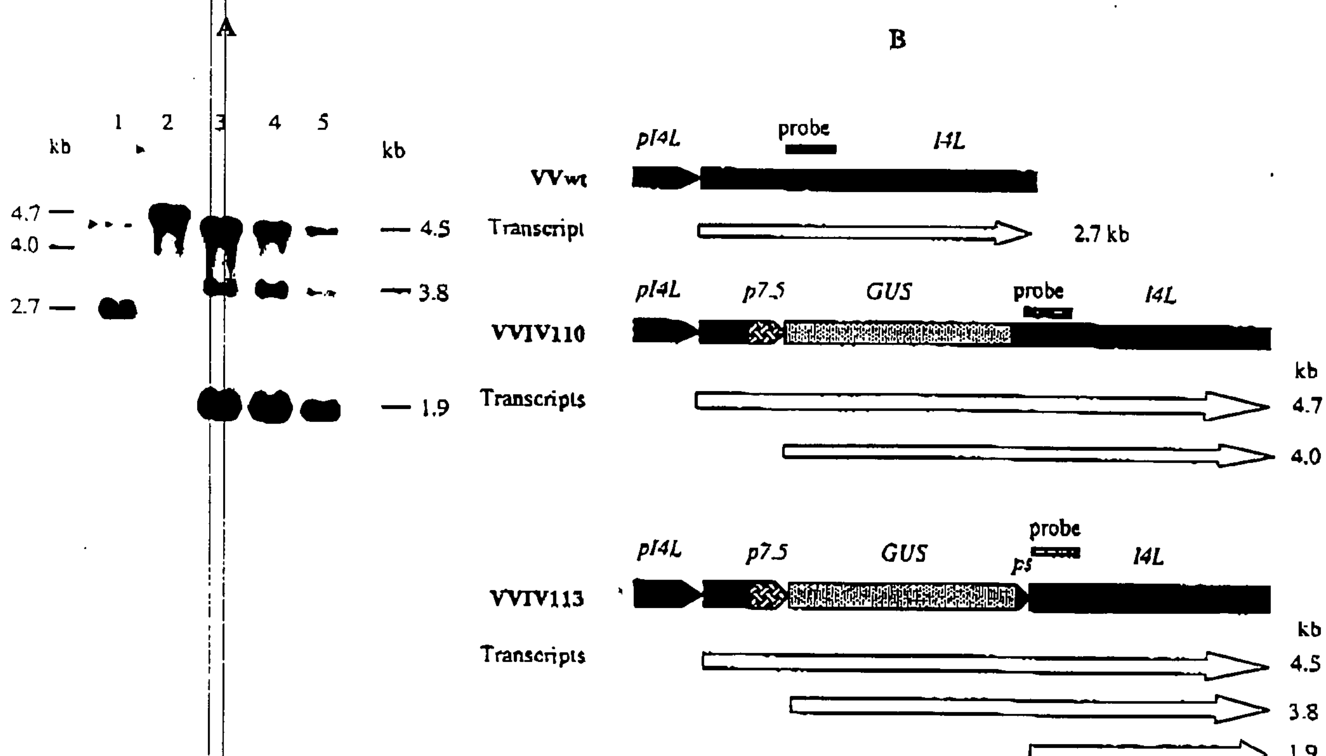


Fig. 3. Northern analysis of the *I4L* transcripts from the various re-VV (A) and a schematic representation showing their predicted sizes (B). A: Northern blot hybridisation of 5 μg of total RNA isolated from BHK21 cells infected with different VV. Lanes: 1, VV Copenhagen strain; 2, VVIV110; 3, VVIV113; 4, VVIV113 NP; 5, VVIV113 NP/P. The arrowheads in lanes 1 and 2 show the position of the *I4L* readthrough transcripts. The approximate molecular weights of RNA transcripts are indicated on the margins. Methods: Total RNA was isolated from BHK21 cells infected for 6 h with the various viruses in the presence of cycloheximide (0.1 mg/ml). From these RNA extracts, 5 μg was electrophoresed on a 1.2% agarose gel (a standard formaldehyde/MOPS gel system) and then Northern blotted onto a positively charged nylon membrane (Boehringer). Hybridisation was carried out with a DIG-labelled antisense *I4L* (*Kpn*I-*Xho*I region) RNA probe (RNA labelling kit, Boehringer). Hybridisation, post-hybridisation washes and the detection procedure were carried out as described in the p... detection kit ... represents ... of th... RNA transcripts. VVIV113 represents also ...

in front of the *NP* and *P* genes (see Fig. 2C). Inter or intra chromosomal recombination between the *p7.5*-promoter in front of *GUS* with either the one in front of *NP* or *P* genes would result in an inversion of the orientation of the intervening sequences. This would generate new *Hin*dIII fragments whose predicted sizes would be 6.0 and 6.6 kb, respectively (Fig. 2A), which would hybridise to the *GUS* and *I4L* probes. Fragments with the expected sizes were actually found as illustrated in Fig. 2B(i) and (ii), lanes 4 and 5. Thus, the presence of two *p7.5*-promoters led to one extra *Hin*dIII fragment and the presence of three *p7.5*-promoters led to two extra *Hin*dIII fragments. Although these re-viruses contained a mixed genomic population with variable orientation of internal sequences they still displayed homogenous phenotypic properties such as the ability to yield 100% blue plaques, encode faithfully the NP and P proteins (results not shown) and induce the synthesis of the predicted transcript from the synthetic promoter (see Fig. 3). The re-viral genomes in which deletions of essential VV sequences have occurred (for instance via recombination between the *p7.5*-promoters upstream from the *P* and *NP* genes) would of course be non viable and diluted out during viral multiplication.

(e) Conclusions

(*1*) Vectors pIV110 and pIV113 were constructed that allow transfer of the *GUS* gene into the *I4L* locus of VV. New viruses express GUS activity enabling blue plaque selection. GUS blue plaque selection to purify viral recombinants in our hands has been very reliable and convenient, only involving an agarose overlay with XGluc.

(*2*) The *GUS* expression cassette did not interfere with transcription from promoters positioned downstream, which is a convenient position to insert a foreign gene of interest.

(*3*) Multiple homologous promoter sequences inserted into different loci of VV do not produce phenotypically unstable viral recombinants even though recombination between the homologous sequences does take place. Recently, we have been able to construct a VV recombinant containing four *p7.5*-promoters with no adverse consequences on phenotypic stability. The methods we have employed should be useful for further development of VV as a polyvalent vector.

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Appendix IV

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Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence

H. Meyer,* G. Sutter† and A. Mayr

Institute of Medical Microbiology, Infectious and Epidemic Diseases, Veterinary Faculty, Ludwig-Maximilians Universität, Veterinärstrasse 13, D-8000 München 22, Germany

Different passages of the vaccinia virus strain Ankara (CVA wild-type) during attenuation to MVA (modified vaccinia virus Ankara) have been analysed to detect alterations in the genome. Physical maps for the restriction enzymes *Hin*dIII and *Xho*I have been established. Six major deletions relative to the wild-type strain CVA could be localized. They reduce the size of the entire genome from 208 kb (CVA wild-type) to 177 kb for the MVA strain. Four deletions occurred during the first 382 passages and the resulting variant (CVA 382) displays an attenuated phenotype similar to that of the MVA strain. The deletions are located in both terminal fragments, affect two-thirds of the host range gene K1L and eliminate 3·5 kb of a highly conserved region in the *Hin*dIII A fragment. During the next 190 passages leading to MVA two additional deletions appeared. Again, one is located in the left terminal fragment, and the other includes the A-type inclusion body gene. Neither of the deletions appear to participate in further attenuation of the virus. Rescue of the partially deleted host range region with the corresponding wild-type DNA restored the ability of the attenuated strains MVA and CVA 382 to grow in some non-permissive tissue cultures. Nevertheless, the complete host range of the wild-type strain was not recovered. Also, plaque-forming behaviour and reduced virulence were not influenced. From the data presented it may be concluded that the partially deleted host range gene is not solely responsible for attenuation.

Introduction

The use of vaccinia viruses as carriers for foreign genes has led to a renewed interest in vaccinia virus research (Moss, 1985). Owing to side-effects observed during the smallpox eradication campaign there are strong concerns about introducing recombinant vaccinia viruses into human vaccination programmes (Kaplan, 1989). Therefore recent studies have focused on so-called strongly attenuated vaccinia virus strains and on virus attenuation. Deletion or insertion of certain DNA sequences, as well as inactivation of single genes have been used to demonstrate their influence on virulence (Flexner *et al.*, 1987; Kotwal *et al.*, 1989; Rodriguez *et al.*, 1989). Mutants of vaccinia virus with a thymidine kinase-negative (TK^-) or small plaque phenotype exhibited a marked decrease in virulence (Buller *et al.*, 1985; Gong *et al.*, 1989). Also the generation of deletions at the left end of the genome contributes to reduced pathogenicity (Dallo & Esteban, 1987; Kotwal & Moss, 1988; Buller *et al.*, 1988).

However, none of these viruses has been tested extensively in a large number of animals. Altenburger *et al.* (1989) investigated the highly attenuated and well characterized strain MVA (modified vaccinia virus Ankara), which had been attenuated by Mayr *et al.* (1975) by more than 570 serial passages in primary chick embryo fibroblasts (CEFs). The host range of this strain is severely restricted as it replicates only in CEFs. MVA has been proven to be avirulent in a variety of animals even under immunosuppression (for review see Mayr *et al.*, 1978; Stickl *et al.*, 1974). It has been used without complications in primary vaccinations in over 120000 humans (Mayr & Danner, 1979). Three major deletions, relative to vaccinia virus Western Reserve, have been identified so far (Altenburger *et al.*, 1989). One affects two-thirds of the 32K human host range gene; this gene is called the K1L gene following recommended nomenclature (Rosel *et al.*, 1986). The other deletions are located in the left and right terminal fragments.

In this study we establish and compare physical maps (*Hin*dIII, *Xho*I and *Sma*I) of MVA (574 passages), a precursor CVA 382 (382 passages) and the original wild-type virus CVA (two passages on CEFs). Marker rescue experiments were conducted to evaluate whether the

† Present address: Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892, U.S.A.

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partially deleted host range gene is responsible for the attenuated phenotype.

Methods

Viruses and cells. Vaccinia viruses used for this study were the reference strain Elstree and the second, 382nd and 574th passage of vaccinia virus Ankara on CEFs (Mayr *et al.*, 1975). In the text they are referred to as CVA wild-type, CVA 382 and MVA respectively. Cell cultures used were: CEFs, chick fibroblast LSCC-H-32 (a gift of O. R. Kaaden, Munich, Germany), bovine embryonic lung (BEL) cells and Madin-Darby bovine kidney (MDBK) cells, the human HeLa, HRT 18, MRC 5 and Hep-2 cells, the monkey cell lines Vero and MA 104, rabbit kidney (RK_{13}), equine dermal (E-derm), mouse DBT cells and Madin-Darby canine kidney (MDCK) cells. The cells were grown as monolayers in Eagle's basal medium (EMEM) supplemented with 5% foetal bovine serum.

Virus propagation and purification. For the preparation of viral stocks s were infected with the appropriate plaque-purified (three times) virus (1 p.f.u. per cell) and incubated for 2 to 3 days at 37 °C. The virus was liberated from cells by freezing and thawing and brief sonication followed by low speed centrifugation to remove cellular debris. Virus stocks were titrated on CEFs, and stored in aliquots at −70 °C. Virus was purified as described by Joklik (1962) with some modifications (Czerny & Mahnel, 1990). The host range of CVA wild-type and the attenuated MVA was compared with the recombinant viruses rec.MVA and rec.CVA 382 by infecting cell monolayers at a multiplicity of 0·05 p.f.u. per cell. Adsorption of virus was allowed to occur for 45 min at 37 °C. After removal of the inoculum, the cell monolayer was washed once with EMEM and incubated with fresh medium (3% FCS in EMEM) at 37 °C. At 0, 48 and 72 h post-infection (p.i.) virus was harvested by freezing and thawing and brief sonication. Virus obtained was titrated on CEFs.

Cloning reagents. Viral DNA was isolated from purified virions and cleaved with restriction enzymes purchased from Boehringer Mannheim and used as specified by the manufacturer. Physical maps for the restriction endonucleases *Hin*dIII, *Xho*I and *Sma*I were established by Southern blot hybridization (Maniatis *et al.*, 1982) using the Digoxygenin labelling and detection system (Boehringer Mannheim). For restriction site mapping, sequences of CVA wild-type, CVA 382 and 'A were cloned by standard procedures into the vector pTZ19R (. harmacia/LKB) using T4 DNA ligase [Gibco/Bethesda Research Laboratories (BRL)]. Transformation of competent *Escherichia coli* bacteria, strain DH5α (Gibco/BRL), was done according to Hanahan (1983). Plasmid DNA was isolated by the procedure of Birnboim & Doly (1979).

Marker rescue. The 5·2 kb *Eco*RI fragment of CVA wild-type, spanning the K1L host range gene (Gillard *et al.*, 1985), was cloned. Marker rescue experiments were done on the E-derm cell line which is non-permissive for the attenuated strains MVA and CVA 382. Subconfluent cells, infected with 0·05 p.f.u. of either MVA or CVA 382 were transfected with calcium phosphate-precipitated plasmid DNA (pTZ 5.2) according to Graham & van der Eb (1973). After 90 min of adsorption fresh medium was added to the cells and incubation was continued for 3 to 5 days. Recombination events could be detected easily by a developing c.p.e. After plaque purification, DNA of the recombinant viruses rec.MVA and rec.CVA 382 was analysed with *Hin*dIII.

Mouse experiments. For the experiments, approximately 6-week-old female NMRI mice and 2- to 3-day-old baby mice were used. Mice were inoculated either intraperitoneally (i.p.) with 3×10^{n} or intracranially (i.c.) with 2×10^{n} $TCID_{50}$, baby mice with 1×10^{5} and 1×10^{4} respectively. Virus strains used were MVA, CVA wild-type and rec.MVA.

Results

Mapping of six major deletions

To demonstrate possible alterations of the genome during continuous propagation we compared the *Hin*dIII restriction profiles of CVA wild-type, CVA 382 and MVA (Fig. 1*a* and *b*). Compared to CVA wild-type DNA a slightly higher mobility of the largest *Hin*dIII A fragments could be observed in CVA 382 and more prominently in MVA. Additionally the fragments B and C migrated faster in CVA 382. Compared to CVA 382 the size of the MVA B fragment remained constant, whereas the C fragment was again reduced in size. Regarding the smaller fragments (Fig. 1*b*), two bands (2·1 and 1·5 kb) of CVA wild-type were missing in CVA 382 but an additional fragment (1·0 kb) appeared. The existence of a recently identified 0·3 kb *Hin*dIII fragment located in the central part of the genome could be demonstrated for CVA wild-type, CVA 382 and MVA in a polyacrylamide gel (data not shown). To determine the exact size of the entire genome we isolated the largest *Hin*dIII and *Xho*I fragments from the gel and cleaved them with *Xho*I, *Hin*dIII or *Eco*RI (data not shown). Using M_r standards and a digitizer-aided computer program (Microgenie, Beckman) the length of the entire genomes adds up to 208 (CVA wild-type), 188 (CVA 382) and 177 (MVA) kb. *Hin*dIII, *Xho*I and *Sma*I genomic maps of CVA wild-type, CVA 382 and MVA were determined by cross-hybridizations (Fig. 2). The location and size of six major deletions is indicated by arrowheads. There are four deletions in the CVA 382 genome relative to CVA wild-type. Deletions I (2·9 kb) and IV (10·2 kb) are located at the left (*Hin*dIII C) and right (*Hin*dIII B) end of the viral genome. This could be shown by cross-hybridizations using CVA wild-type *Xho*I fragments F, H, M and N as well as CVA 382 *Xho*I I, E and F (Fig. 2). Deletion II has already been described (Altenburger *et al.*, 1989) and affects a 55K polypeptide as well as two-thirds of the 32K human host range gene product. Deletion III is located at the right-hand side of the *Hin*dIII A fragment and leads to the loss of 3·5 kb including the only *Sma*I restriction site. Detailed restriction site mapping of this region was carried out using cloned *Eco*RI–*Hin*dIII fragments from the right side of *Hin*dIII A of CVA wild-type and CVA 382 (Fig. 3). Although CVA 382 and MVA display a haemagglutinin-negative phenotype (HA^-), the haemagglutinin gene (Shida, 1986) does not seem to be affected by this deletion.

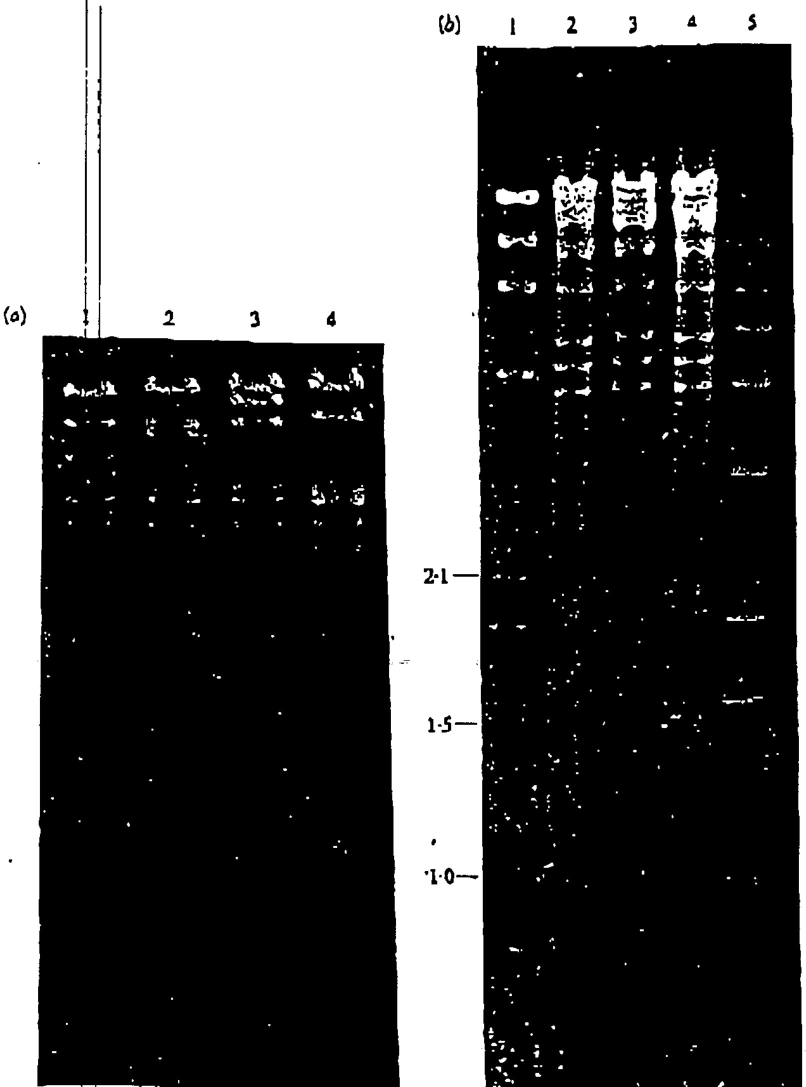

Fig. 1. (*a*) Electrophoresis of *Hin*dIII digests of DNAs from vaccinia virus MVA (lane 1), CVA 382 (lane 2), CVA wild-type (lane 3) and vaccinia virus Elstree (lane 4). The digests were electrophoresed on a 0·5% agarose gel. (*b*) Electrophoresis of *Hin*dIII digests of DNAs from CVA 382 (lane 2), CVA wild-type (lane 3) and vaccinia virus Elstree (lane 4). M_r standards: λ *Hin*dIII DNA (lane 1) and kb size ladder (lane 5). The digests were electrophoresed on a 1·2% agarose gel.

After a further 190 passages leading to MVA, two additional deletions occurred, one being located again in the *Hin*dIII C fragment (deletion V: 4·7 kb), the other in the middle of the *Hin*dIII A fragment (deletion VI: 3·8 kb). *Eco*RI clones of the *Hin*dIII C fragment of MVA and CVA 382 were used to map deletion V. This deletion reduces the size of the 5·8 kb *Eco*RI fragment, located at the very right end side, to 1·1 kb (Fig. 4). Deletion VI eliminates sequences of two adjacent *Eco*RI fragments (3·6 and 2·0 kb) and results in the formation of a new 1·8 kb fragment (Fig. 4), thus deleting nearly the entire A-type inclusion body gene (Funahashi *et al.*, 1988).

Marker rescue of the partially deleted host range gene

Non-permissive E-derm cells were infected with either MVA or CVA 382 and then transfected with plasmid pTZ 5.2, as we had hoped that by directly transfecting a non-permissive cell line only recombinant viruses would be able to replicate, allowing easy isolation. In fact, accumulation of rounded cells could be seen 2 to 4 days after infection. Subsequently, virus was isolated and after plaque purification named rec.MVA and rec.CVA 382, respectively. However cells infected with MVA or CVA 382 alone displayed no c.p.e., even after five

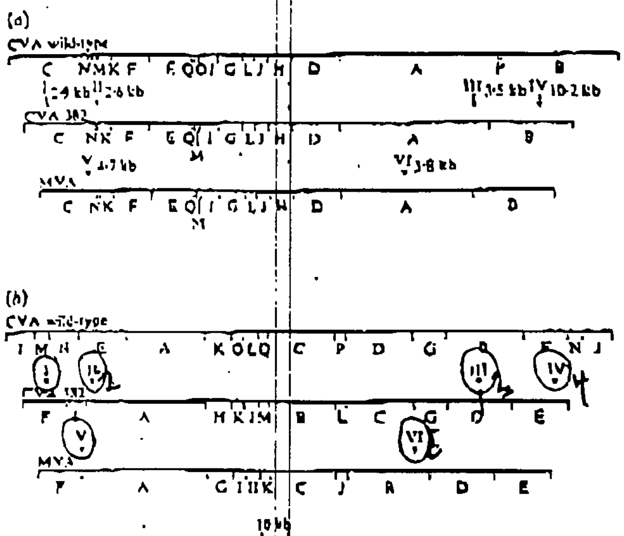

Fig. 2. Physical arrangement of (*a*) *Hin*dIII and (*b*) *Xho*I genome DNA fragments of vaccinia virus CVA wild-type, CVA 382 and MVA. Fragments are labelled alphabetically according to size. Location and size of six major deletions (I to VI) are indicated by arrowheads.

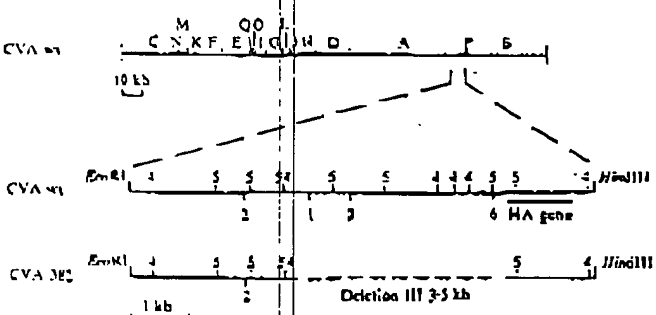

Fig. 3. *Hin*dIII restriction map of CVA wild-type DNA. The cloned 7·8 kb *Eco*RI–*Hin*dIII fragment of CVA wild-type containing the haemagglutinin gene (HA gene) is compared to the corresponding 3 kb *Eco*RI–*Hin*dIII clone of CVA 382. Dashed lines indicate the location of deletion III. Restriction sites are abbreviated as follows: 1, *Sma*I; 2, *Bam*HI; 3, *Pst*I; 4, *Cla*I; 5, *Acc*I; 6, *Sal*I.

continuous passages. To identify the rearranged fragments, DNA from rec.MVA and rec.CVA 382 was cleaved with *Hin*dIII (Fig. 5, data for CVA 382; data for rec.CVA 382 are not shown). Gel electrophoresis demonstrated that the deletion had been restored since both recombinant viruses possessed the expected *Hin*dIII 2·1 and 1·5 kb fragments characteristic of CVA wild-type.

Phenotypic characterization

The host range was tested on 14 different cell lines (Table 1). CVA wild-type virus was able to multiply in a broad range of different cell lines. Seventy-two hours p.i., titres

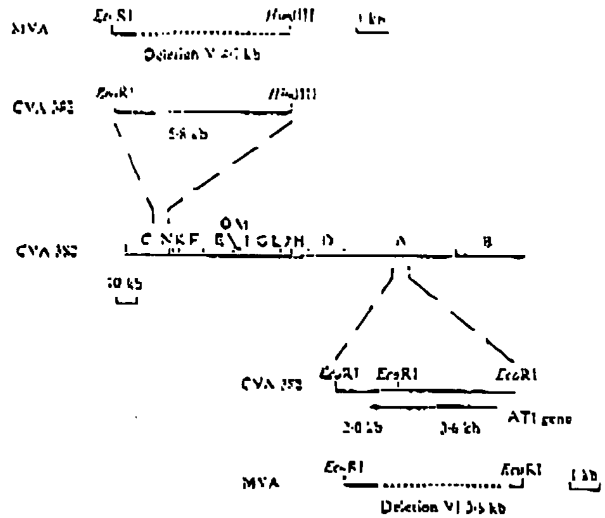

Fig. 4. *Hin*dIII restriction map of CVA 382 DNA. Deletions V and VI in the MVA relative to the CVA 382 genome are indicated by dashed lines.

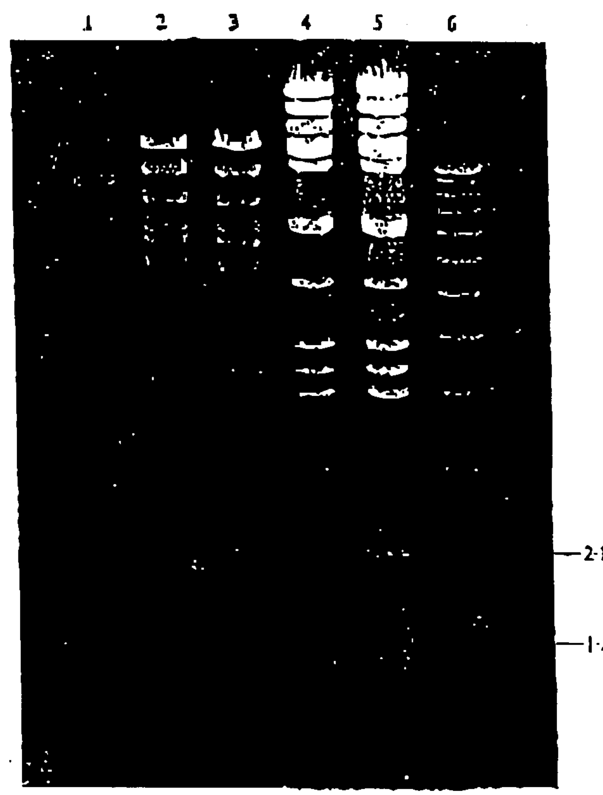

Fig. 5. *Hin*dIII digests of DNA from vaccinia virus MVA (lanes 2 and 4) and recombinant rec.MVA (lanes 3 and 5) in a 0·6% agarose gel. The digests were electrophoresed for 36 h (lanes 1, 2 and 3) and 18 h (lanes 4, 5 and 6). M_r standards: λ *Hin*dIII DNA (lane 1) and kb size ladder (lane 6).

measured on CEFs had usually increased 100- to 3000-fold compared to titres at the end of the adsorption period. However, CVA wild-type was not able to

'able 1. *Multiplication of vaccinia virus CVA wild-type, {VA and recombinant viruses rec.MVA and rec. CVA 382 ı different cell lines*

Cell type	CVA wt	MVA	rec. MVA	rec. CVA 382
:hick				
CEF	1778*	15335	16125	10000
LSCC-H-32	100	316	ND†	ND
{uman				
HeLa	1334	0·6	4·2	4
MRC 5	1778	0·4	1·4	4
HRT 18	316	0·8	7·5	8
Hep-2	3162	0·2	42	56
Aonkey				
Vero	1000	4	56	56
MA 104	316	237	177	133
Rabbit				
RK_{13}	237	0·6	3162	4217
:quine				
[illegible]	422	0·8	1000	1778
Jovine				
BEL	316	4	0·3	0·2
MDBK	2	0·6	ND	ND
Canine				
MDCK	1334	0·2	1	6
Aouse				
DBT	0·6	0·4	ND	ND

* The values represent the ratio of the titres obtained after 72 h p.i. over the titres in the cultures at the end of the adsorption period.
† ND, Not determined.

replicate in mouse DBT or bovine MDBK cells. In contrast, the attenuated strain MVA was restricted to primary and permanent chick fibroblasts and to the monkey cell line MA 104. Three days after infection virus yields were comparable to the CVA wild-type strain or in the case of the CEFs 10 times higher. In parallel the same experiments were done for the recombinant viruses rec. MVA and rec. CVA 382. Reintroduction of the K1L and the adjacent gene, encoding a 55K protein, extended the host range of the recombinant viruses. Virus yield obtained on non-permissive E-derm and RK_{13} cells were even higher than the yield of CVA wild-type. Vero and Hep-2 cells were also permissive for multiplication, albeit to a limited extent. The existence of three kinds of infections with the recombinant viruses, i.e. permissive, semi-permissive or non-permissive, is clearly illustrated in comparative growth curves (Fig. 6).

After infection, pocks produced on the chorioallantoic membrane (CAM) of embryonated eggs by CVA wild-type were large with a deep, central necrosis. On the other hand pocks produced by MVA, CVA 382 as well as by the recombinant viruses rec. MVA and rec. CVA 382 were small, proliferative and without necrosis.

Baby mice infected i.p. or i.c. with CVA wild-type died 2 to 3 days after infection (Table 2). In contrast, all

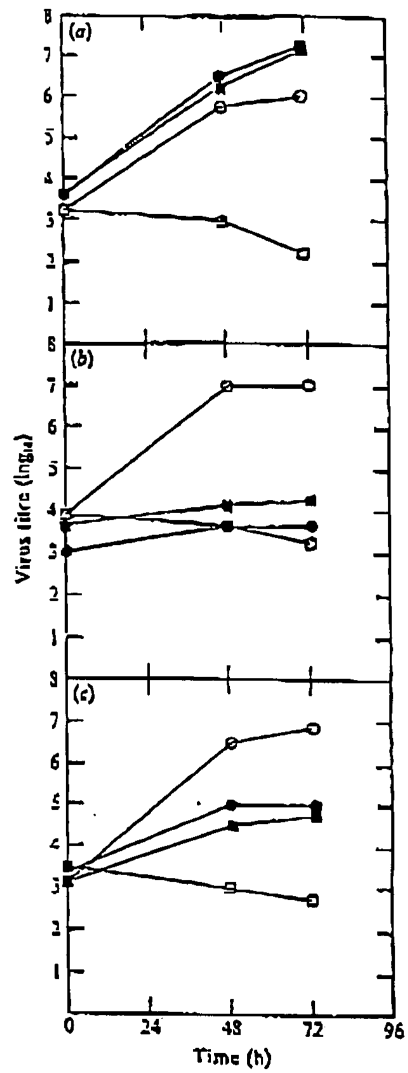

Fig. 6. Multiplication of vaccinia virus CVA wild-type (○) and MVA (□) as well as recombinants, rec. MVA (■) and rec. CVA 382 (●) in three different cell types: (*a*) RK_{13}; (*b*) HeLa; (*c*) Hep-2.

Table 2. *Virulence of vaccinia virus CVA wild-type, MVA and recombinant rec.MVA after i.p. or i.c. inoculation**

	Virus	No. of animals	Application i.c. Ill	Application i.c. Died	Application i.p. Ill	Application i.p. Died
Baby mice	CVA wt	30	30/30	30/30	30/30	30/30
	MVA	15	0/15	0/15	0/15	0/15
	rec. MVA	30	0/30	0/30	0/30	0/30
Mice 6-week	CVA wt	10	10/10	3/10	10/10	0/10
	MVA	10	0/10	0/10	0/10	0/10
	rec. MVA	30	0/30	0/30	0/30	0/30

* NMRI mice were inoculated either i.p. with 3×10^6 or i.c. with 2×10^4 $TCID_{50}$, baby mice with 1×10^5 and 1×10^4 respectively.

animals infected with either MVA or rec. MVA survived without any clinical symptoms. In adult mice all animals infected i.c. with wild-type virus showed marked signs of illness and three out of 10 died. After i.p. infection all animals developed a generalized infection with multiple pocks starting 7 days after infection. Animals infected with MVA or rec. MVA never showed any signs of illness.

Discussion

In this study we compared the genomes of the strongly attenuated strains MVA and CVA 382 with the ancestral CVA wild-type strain. We provide evidence that during attenuation several alterations in the genome have occurred. Owing to limitations of the technology used, deletions smaller than 0·3 kb are difficult to detect and point mutations would be missed. However, the size of six major deletions identified so far adds up to about 30 kb. In other words, nearly 15% of the entire genome is non-essential for virus replication *in vitro*. Mutants of vaccinia virus strains have been described with as much as 21·7 kb of DNA deleted from the left terminus (Drillien *et al.*, 1981; Perkus *et al.*, 1989). The sizes we determined for the genomes of CVA wild-type and vaccinia virus Elstree (208 and 200 kb respectively) have been obtained after subcleavage of isolated large *Hin*dIII and *Xho*I fragments. Our values are higher than data from other authors (Esposito & Knight, 1985; Mackett & Archard, 1979). They determined the size by using comigrating λ *Hin*dIII fragments as an M_r marker only. However, our results are confirmed by sequence data published recently (Goebel *et al.*, 1990) and match those of Bostock (1988) who determined genomic sizes by pulsed-field electrophoresis.

Mapping of the deletions and marker rescue experiments are first steps to defining functions involved in attenuation. During the first 382 passages four major deletions could be identified, decreasing the size of the CVA wild-type genome from 208 to 188 kb. Two deletions (I and IV) are located in the terminal fragments. Neither could be mapped precisely because of transposition of sequences (unpublished data). This will be the subject of further research. The presence of long stretches of non-essential DNA near the two ends of the genome is still an unexplained structural feature of the orthopoxviruses (Gangemi & Sharp, 1976; Panicali *et al.*, 1981; Paez *et al.*, 1985). It is assumed that these sequences encode a variety of proteins which interact with the host (Moyer *et al.*, 1980; Moss *et al.*, 1981; Pickup *et al.*, 1986; Kotwal & Moss, 1988; Smith *et al.*, 1989).

Therefore deletions or mutations can attenuate virus pathogenicity (Buller *et al.*, 1985; Dallo & Esteban, 1987). Additionally both terminal regions can readily undergo complex sequence rearrangements or transpositions during continuous propagation in cell cultures (Moyer *et al.*, 1980; Esposito *et al.*, 1981; Pickup *et al.*, 1984). Deletion II has been described for the MVA strain by Altenburger *et al.* (1989): a 2·6 kb deletion eliminates most of the two adjacent 2·1 and 1·5 kb fragments resulting in a new 1·0 kb fragment. From our findings it is clear that this deletion occurred during the first 382 passages and remained stable throughout the next 190 passages leading to MVA. During attenuation, 3·5 kb from the right side of the *Hin*dIII A fragment have been eliminated. Although no deletions or mutations within this region have been described so far, it is non-essential for replication *in vitro*. According to Goebel *et al.* (1990), in the vaccinia virus strain Copenhagen these sequences contain three proposed open reading frames but their function has not been defined. Although CVA 382 displays an HA⁻ phenotype in contrast to CVA wild-type, the deletion does not affect the haemagglutinin gene. However, an HA⁻ phenotype can be caused by a single point mutation (Shida, 1986).

During the next 190 passages two deletions occurred and reduced the size from 188 to 178 kb for the MVA strain. Once again the left end of the genome was affected. Whether this deletion contributes to a further decrease in virulence cannot be evaluated because CVA 382 displays the same attenuated phenotype as MVA. The second deletion affects the gene encoding the major protein of the A-type inclusion body (ATI). Despite the morphological absence of ATIs in vaccinia virus compared to cowpox, monkeypox or ectromelia viruses, an antigenically related protein is induced (Kitamoto *et al.*, 1986). The ATI gene of cowpox virus has been mapped and sequenced recently (Funahashi *et al.*, 1988). In vaccinia virus, approximately 400 bp starting at the initiation codon of the vaccinia virus ATI gene equivalent have been sequenced by Patel *et al.* (1988). Compared to the corresponding sequences of cowpox virus there are only two bases which are different. From several conserved restriction enzyme sites the authors assume that both ATI genes are probably located in the same position. Although the ATI gene is one of the most strongly expressed genes it is not essential for replication in cell culture (Patel *et al.*, 1988). Mature viruses are occluded into the ATI and it has been assumed that such bodies protect the virus during dissemination from animal to animal. Our findings are confirmed by the use of a monoclonal antibody which was raised against the major protein of the ATI and reacts with CVA 382 but not with MVA (C. P. Czerny, personal communication).

To understand more precisely the significance of a restricted host range in virulence, marker rescue experi-

ients of the host range phenotype were undertaken. For ur experiments we used an *Eco*RI fragment which upon isertion into the TK gene locus of the Copenhagen host inge mutant restored a wild-type phenotype (Gillard *et l.*, 1985). According to Altenburger *et al.* (1989) this ragment overlaps the entire host range deletion of the 1VA strain as well as some undeleted sequences anking both ends. In our experiments we transfected 1VA- or CVA 382-infected cells with the corresponding ·2 kb CVA wild-type fragment. It has been shown that nsertion of the K1L gene which leads to an increased ost range can be used as a selection system for ecombinant viruses expressing foreign genes (Perkus *et l.*, 1989). However, our results indicate that insertion of he wild-type host range gene did not restore the wild-ype phenotype; in fact the recombinants rec. MVA and er CVA 382 combined properties of the wild-type and h attenuated strain. On the one hand their yield on a ew cell lines was comparable to that of the CVA wild-ype, and on the other hand c.p.e., plaque morphology on he CAM and complete avirulence for mice were identical to the attenuated strains MVA and CVA 382.

This strongly implies that one or more other genes determining the host range of CVA wild-type are affected. One possible gene is the recently identified 'second' host range gene (ORF C7L) which has been described for the vaccinia virus Copenhagen strain by Perkus *et al.* (1990). Either C7L or the K1L host range gene is necessary and sufficient to allow replication. From hybridization data (G. Sutter, unpublished results) the C7L gene seems to be conserved in the MVA strain but it is not yet clear whether it is functionally active. In cowpox virus a 'third' host range gene has been identified which is responsible for multiplication in Chinese hamster ovary cells and which is independent of the K1L gene (Spehner *et al.*, 1988). From transfection experiments all three host range genes appear to play overlapping roles in determining replication competence on various cell lines.

From the data obtained, marker rescue of the host range gene had no effect on the small plaque size phenotype and on the pock morphology of the attenuated strains. One might speculate whether reduced virulence is caused by a restricted host range and subsequently by the inability to multiply *in vivo* or whether it is related to the altered phenotype in cell cultures and on the CAM or a combination of both.

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Appendix V

page 1359 from the textbook Fields et al, Virology (1996) 3rd edition, Lippincott-Raven Publishers, Philadelphia

and has several regions of homology with the NP of influenza A and B viruses [reviewed in (205)].

The Hemagglutinin Protein and its Gene

The hemagglutinin was originally named because of the ability of the virus to agglutinate erythrocytes (136,251) by attachment to specific sialic glycoprotein receptors. Today we know HA has three major roles during the influenza virus replicative cycle:

1. HA binds to a sialic acid-containing receptor on the cell surface, bringing about the attachment of a virus particle to the cell.
2. Hemagglutinin is responsible for penetration of the virus into the cell cytoplasm by mediating the fusion of the membrane of the endocytosed virus particle with the endosomal membrane, the consequence of which is that viral nucleocapsids are released into the cytoplasm.
3. Hemagglutinin is the major antigen of the virus against which neutralizing antibodies are produced and influenza virus epidemics are associated with changes in its antigenic structure.

Hemagglutinin is encoded by RNA segment 4, and the polypeptide is synthesized in the endoplasmic reticulum (ER) as a single polypeptide HA_0 (M_r ~76,000). An N-terminal signal peptide targets the nascent chain to the ER membrane and is cleaved by signal peptidase, which makes HA a prototype type I integral membrane protein (Fig. 3). The native protein for the H3 subtype consists of an ectodomain of 512 residues, a carboxyl-terminal proximal transmembrane domain of 27 residues, and a cytoplasmic tail of 10 residues (377). Hemagglutinin is co(post)-translationally modified by the addition of up to seven oligosaccharide chains added to the ectodomain, and three palmitate residues are added by a thioether linkage to the three C-terminal proximal cysteine residues (264,265,336). The HA spike glycoprotein is a homotrimer (401,403,408) of noncovalently linked monomers. An x-ray structural determination of both the HA trimer (395, 396,404,408) and the trimer bound to a receptor analogue, sialyl lactose, has been obtained (394), as well as a proteolytic fragment of the low pH conformation (46). Depending on the virus strain, host-cell type, and growth conditions, HA is uncleaved or cleaved into two disulfide-linked chains HA_1 (M_r ~47,000) and HA_2 (M_r ~29,000). Cleavage is required for the virus to be infectious and is thus a critical determinant in pathogenicity and in the spread of infection [reviewed in (205)]. The newly liberated N-terminus of HA_2 (fusion peptide) is hydrophobic, is highly conserved in HAs of different influenza virus strains, and has been implicated as an essential participant in HA fusion activity.

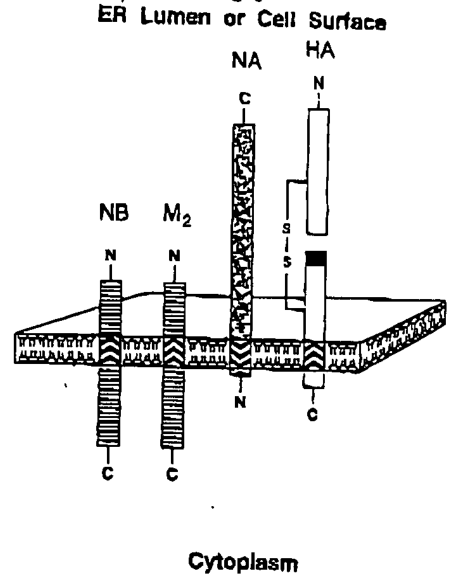

FIG. 3. Schematic representation of the orientation of the influenza virus integral membrane proteins HA, NA, M_2, and NB. See text for details of the individual proteins.

RNA Segment 4: Gene Structure and HA Amino Acid Sequence

The first gene of influenza virus to have its nucleotide sequence determined was HA (305). Since then, the nucleotide sequences of RNA segment 4 of all 14 known HA antigenic subtypes and many variants within a subtype have been determined [reviewed in (205,277)]). RNA segment 4 ranges from 1,742 to 1,778 nucleotides and encodes a polypeptide of 562 to 566 residues. The HA_1 chain is from 319 to 326 residues, and HA_2 is from 221 to 222 residues. Depending on the subtype, the number of residues lost on proteolytic cleavage between HA_1 and HA_2 ranges from one to six residues. For the A/Aichi/68 H3 subtype, for which the x-ray crystallographic structure was obtained, the cleaved signal sequence contains 16 residues, native HA_1 contains 328 residues, and HA_2 contains 221 residues (377). A single arginine residue is lost on proteolytic cleavage of HA_0, suggesting that two enzymes, a trypsin-like enzyme and an exopeptidase of the carboxypeptidase B type, are involved in activation of HA (82,101). The deduced amino acid sequence of the influenza B/Lee/40 HA has 24% homology to HA_1 and 39% homology to HA_2 of A/PR/8/34, suggesting a close evolutionary relationship between influenza A and B virus HAs (202,203).

Three-Dimensional Structure of HA

Although intact HA "spikes" can be isolated from purified influenza virions and infected cells by detergent sol-

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